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Targeting sequences of the two major peroxisomal proteins in the methylotrophic yeast *Hansenula polymorpha*

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Summary. Dihydroxyacetone synthase (DAS) and methanol oxidase (MOX) are the major enzyme constituents of the peroxisomal matrix in the methylotrophic yeast *Hansenula polymorpha* when grown on methanol as a sole carbon source. In order to characterize their topogenic signals the localization of truncated polypeptides and hybrid proteins was analysed in transformed yeast cells by subcellular fractionation and electron microscopy. The C-terminal part of DAS, when fused to the bacterial β -lactamase or mouse dihydrofolate reductase, directed these hybrid polypeptides to the peroxisome compartment. The targeting signal was further delimited to the extreme C-terminus, comprising the sequence N-K-L-COOH, similar to the recently identified and widely distributed peroxisomal targeting signal (PTS) S-K-L-COOH in firefly luciferase. By an identical approach, the extreme C-terminus of MOX, comprising the tripeptide A-R-F-COOH, was shown to be the PTS of this protein. Furthermore, on fusion of a C-terminal sequence from firefly luciferase including the PTS, β -lactamase was also imported into the peroxisomes of *H. polymorpha*. We conclude that, besides the conserved PTS (or described variants), other amino acid sequences with this function have evolved in nature.

Key words: Peroxisomes – Targeting signals – Yeast – *Hansenula polymorpha*

Introduction

Organellar proteins that are synthesised in the cytosol of a eukaryotic cell generally contain *cis*-acting signals for sorting and membrane translocation. Such sequences

are well characterised on polypeptides destined for mitochondria (Attardi and Schatz 1988; Pfanner and Neupert 1990; Verner and Schatz 1988), chloroplasts (Ellis and Robinson 1987; Schnell et al. 1991; Smeekens et al. 1990), the endoplasmic reticulum (ER), i.e. secretory, lysosomal and luminal ER proteins (Blobel 1980; Pelham 1986; Rapoport 1990; Walter and Lingappa 1986; Wiedmann et al. 1987), and the nucleus (Dingwall and Laskey 1986; Silver 1991). Signal sequences of proteins imported into mitochondria, chloroplasts and the ER are generally located at the N-terminus and cleaved off during translocation. Such N-terminal sequences also commonly mediate protein export in bacteria (Schatz and Beckwith 1990; Wickner 1989).

Until recently, little was known about import of proteins into microbodies (peroxisomes, glyoxysomes and glycosomes), another ubiquitous type of organelle in eukaryotic cells (Borst 1989; Kindl 1982; Lazarow and Fujiki 1985; Veenhuis and Harder 1987). Peroxisomal proteins were shown to be synthesised on free polysomes and posttranslationally imported without detectable modification (Goldman and Blobel 1978; Lazarow and Fujiki 1985). The overall import process requires ATP but no membrane potential, when tested in an in vitro system (Imanaka et al. 1987). A peroxisomal targeting signal (PTS) was recently identified at the extreme C-terminus of the *Photinus pyralis* luciferase by expression in mammalian cells (Gould et al. 1989) and also in rat liver acyl-CoA oxidase by in vitro studies (Miyazawa et al. 1989). The PTS was shown to consist of the tripeptide S-K-L-COOH, which was both necessary for import and sufficient to direct cytosolic proteins to peroxisomes. In addition, the PTS was shown to be degenerate to a certain extent and to be functional in the yeast *Saccharomyces cerevisiae*, plants and mammalian cells (Gould et al. 1990). However, a variety of peroxisomal proteins, especially from yeasts, do not show the PTS consensus sequence (Borst 1989; Gould et al. 1989) and therefore alternative sequences must exist. Recently, the rat 3-ketoacyl-CoA thiolase was reported as a first example; it contains a cleavable PTS with a novel primary

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sequence that is located at the N-terminus (Swinkels et al. 1991).

In the present study we attempted to identify PTSs in the methylotrophic yeast *Hansenula polymorpha*. Methylotrophic yeasts are highly attractive for this purpose, since massive proliferation of peroxisomes can readily be induced by manipulation of the growth conditions (Goodman 1984; Roa and Blobel 1983; Roggenkamp et al. 1984, 1975; Veenhuis and Harder 1987). In addition, the cloning of genes coding for peroxisomal enzymes (Janowicz et al. 1985; Ledebøer et al. 1985) and the development of transformation procedures (Cregg et al. 1985; Roggenkamp et al. 1986; Tikhomirova et al. 1986) have recently been achieved. The identification of PTSs should provide an initial basis for a genetic and biochemical dissection of the protein recognition and translocation machinery on the peroxisomal membrane.

We localized functional regions in the cloned genes (Janowicz et al. 1985; Ledebøer et al. 1985) coding for the major peroxisomal proteins dihydroxyacetone synthase (DAS) and methanol oxidase (MOX) by expressing deletions and fusions in transformed yeast cells. It could be demonstrated that both proteins contain short targeting signals at their C-termini with novel primary sequences, capable of routing cytosolic proteins into peroxisomes. Further studies on the localization of fusion proteins and truncated polypeptides of DAS revealed that other parts of this protein and/or its three-dimensional structure are also indirectly relevant for peroxisomal import.

Materials and methods

Strains and media. Amplification of plasmids was done in *Escherichia coli* MB 1000 (*hsrK hsmK lac trp pyrF*) (Stinchcomb et al. 1980), either grown in LB supplemented with 100 µg/ml ampicillin or 50 µg/ml neomycin or in M9 medium with 20 µg/ml tryptophan (Maniatis et al. 1982). Protein translocation studies were performed in strain LR9 (*odc1*), an orotidine-5'-phosphate decarboxylase-deficient mutant (Roggenkamp et al. 1986) of *H. polymorpha* (ATCC 34438). Yeast cells were grown on 0.67% Yeast Nitrogen Base (YNB) without amino acids (Difco) supplemented with carbon sources as indicated.

Plasmid constructions. All expression vectors used in this study are derivatives of the vector pHARS1, which contains an autonomously replicating sequence of *H. polymorpha* and the *URA3* gene of *S. cerevisiae* that complements the *odc1* mutation of strain LR9 (Roggenkamp et al. 1986). The expressed fusion proteins or truncated polypeptides are indicated in brackets (see Fig. 2 for alignment of fusions).

Plasmid pDH3 (f3) was constructed by ligation of a 3.2 kb *Bam*HI-*Eco*RI fragment of the *DAS* gene (Janowicz et al. 1985) into the corresponding sites of pME4b (Fig. 1), which was generated by insertion of a *Bam*HI linker into the *Sal*I site of pME4 (M. Eckart, Dissertation, University of Düsseldorf 1988). Construction of

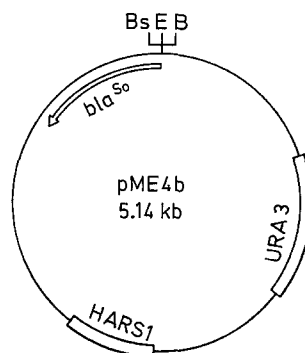


Fig. 1. Structure of pME4b. The plasmid is a derivative of the yeast integration vector YIp5 (Stinchcomb et al. 1980) carrying the *URA3* gene of *Saccharomyces cerevisiae*. The autonomously replicating sequence HARS1 of *Hansenula polymorpha* is inserted into the *Pvu*II site. The plasmid lacks the sequence between the *Sal*I site of the tetracycline resistance gene and the β -lactamase (*bla*) gene up to the 24th amino acid of its coding region resulting in the gene coding for *bla*^{So}. Bs, *Bst*EII; E, *Eco*RI; B, *Bam*HI

plasmid pDH82 (d82) was performed in the same way by insertion of a 4.1 kb *Bam*HI-*Sal*I fragment of the *DAS* gene into the *Bam*HI and filled-in *Eco*RI sites of pME4b. Plasmid pDH147 (f147) was constructed in the same way as pDH82, but instead of the *Eco*RI site the *Bst*EII site of pME4b was used for insertion.

To construct plasmid pDH258 (d258), the internal *Eco*RI-*Bgl*II fragment of the *DAS* gene was excised from the vector pDH131 (H. Hansen, Dissertation, University of Düsseldorf 1990) and the vector was religated after the ends had been blunted with Klenow polymerase. The vector pDH131 carries the whole *DAS* gene as a *Bam*HI fragment (Janowicz et al. 1985) between the *Pvu*I and the *Eco*RV sites of plasmid pHARS1.

Plasmid pDH237 (f237) was constructed in the same way as pDH258, but instead of an internal *DAS* deletion a truncated β -lactamase gene (coding for *bla*^{So} Δ) was inserted into the *Eco*RI site of *DAS*. The β -lactamase fragment spans the region encoding the mature protein except the C-terminal 39 amino acids, as determined by sequencing a *Bal*31 deletion (H. Hansen, Dissertation, University of Düsseldorf 1990), between two *Eco*RI sites. The 5' *Eco*RI site is identical to that in pMEX (Hansen and Roggenkamp 1989) and the 3' *Eco*RI site was created by insertion of a dodecamer *Eco*RI linker into a *Sma*I site of an octamer *Sma*I linker at the *Bal*31 deletion end-point.

In plasmid pDH115 (d115) the *Eco*RI-*Bgl*II β -lactamase fragment in the vector pMEX was replaced by a 2.2 kb *Sma*I-*Bam*HI fragment of the *DAS* gene after filling-in the *Eco*RI site.

Plasmid pDH105 (f105) was constructed as follows: a *Bam*HI linker was inserted into the *Bgl*II site of pMEX and a *Xho*I-*Sal*I fragment encoding the neomycin resistance gene (Beck et al. 1982) was inserted into the *Sal*I site of pMEX, resulting in plasmid pMEK1. The region encoding the mature β -lactamase was replaced by the same β -lactamase fragment that was used for constructing pDH237, but instead of the inserted *Eco*RI linker at the 3' end, it contains a dodecamer *Cla*I linker. A

0.3 kb *ScaI*-*Bam*HI fragment of *DAS* was inserted between this *Cla*I site, after filling in, and the *Bam*HI site.

Plasmid pDH4 (f4) was constructed as for pDH105, but a synthetic double-stranded oligonucleotide of sequence

```
5' -CGCTGATCAAGGCCAAGAAGGGCGGCAAGTC-
GAAGCTGTAA
3' -GCGACTAGTTCCGGTTCTTCCCGCCGTTTCAGCTTC-
GACATTCTAG
      L I K A K K G G K S K L *
```

was inserted instead of the *DAS* fragment. Plasmid pDH11 (f1-2) was constructed in the same way as pDH4, but the synthetic oligonucleotide had the sequence

```
5' -CGGTAAATAAGCTCTAA
3' -GCCATTTATTTCGAGATTCTAG
      V N K L *
```

Plasmid pBM4 (f3-4) was constructed in the same way as pDH4, but the synthetic oligonucleotide had the sequence

```
5' -GCCTTGCCAGATTCTAA
3' -CGGAACGGTCTAAGATTCTAG
      L A R F *
```

In plasmid pDH13 (f13) the *Sal*I-*Bst*EII fragment of pMEK1, carrying the neomycin resistance gene and the *MOX* gene promoter, was changed for a fragment of pDH17, digested with the same enzymes. This fragment of pDH17 encodes the neomycin resistance gene and the *MOX* promoter plus the open reading frame of the *DAS* gene up to the *Bst*EII site at position 339 from the ATG initiation codon (Janowicz et al. 1985). Plasmid pDH17 was generated from pMEX by insertion of the neomycin resistance gene as in the case of pDH105. In addition, the *Eco*RI-*Cla*I fragment carrying the β -lactamase gene was replaced with the open reading frame of the *DAS* gene on a *Bam*HI fragment in plasmid pIX17 (Gödecke et al. 1989).

Plasmid pDH41 (f41) was constructed as follows: plasmid pXIV-2 (provided by G. Schatz) carries an insert of the mouse dihydrofolate reductase (DHFR) coding region bounded by an *Eco*RI and a *Hind*III site as in the vector pDS5/2 (Stueber et al. 1984). A *Cla*I linker was inserted into the *Hind*III site and the *Eco*RI-*Cla*I fragment was ligated into pMEX in place of the *Eco*RI-*Cla*I fragment of the β -lactamase gene of pMEX. The resulting plasmid, pMED, was used for expression of DHFR under control of the *MOX* promoter. It was partially digested with *Acc*I, and after filling-in the ends digestion with *Bgl*III was performed for insertion of the *Sca*I-*Bam*HI fragment of *DAS* as in pDH105. Plasmid pDH8 (f8) was constructed like plasmid pDH41, but the synthetic oligonucleotide used for pDH4 was inserted instead of the *DAS* fragment.

For the construction of plasmid pDHN12 (d12) the *Bam*HI-*Hind*III subclone of the *DAS* gene (Janowicz et al. 1985) was inserted into the multiple cloning site of the vector M13mp19. Oligonucleotide-directed in vitro mutagenesis was performed with phosphorothioate-modified DNA (Taylor et al. 1985) using an in vitro

mutagenesis kit (Amersham). The experimental procedure was carried out according to the manufacturers instructions. An oligonucleotide with the sequence

```
5' -GTTGGTTCGTCGACCGACGC
3' -CAAGCAGCAGCTGGCTGCG
```

was used to generate a *Sal*I restriction site (italicised) 31 bp upstream of the initiation codon of the *DAS* open reading frame (Janowicz et al. 1985). Subsequently, a limited *Sal*I digestion was carried out, the new *Sal*I site was filled-in using Klenow enzyme and a 10mer *Cla*I linker (Pharmacia) was inserted to produce an in-frame ATG initiation codon. The *Cla*I-*Bam*HI fragment encoding the truncated *DAS* gene, after filling-in the *Cla*I site using Klenow enzyme, was inserted into pMEX, after this vector had been cut with *Eco*RI, filled-in using Klenow enzyme and cut with *Bgl*III. The truncated *DAS* gene codes for a polypeptide that lacks the first 12 amino acids at the N-terminus compared with the authentic *DAS*.

DNA sequencing. The dideoxynucleotide chain-terminating procedure (Chen and Seeburg 1985; Sanger et al. 1977) was used with a T7 polymerase sequencing kit (Pharmacia).

Transformation procedures. *E. coli* cells were transformed by the CaCl_2 heat shock method (Maniatis et al. 1982). For transformation of *H. polymorpha*, frozen cells (Klebe et al. 1983) treated with polyethylene glycol were used as described earlier (Roggenkamp et al. 1986) with minor modifications.

Cell fractionation. Cells were grown selectively on YNB plus 3% glycerol and 1% methanol as carbon sources for 36–48 h in a total volume of 500 ml. Cells were converted to protoplasts, gently lysed by osmotic shock and organelle pellets were separated on a 30%–60% sucrose gradient essentially as described earlier (Hansen and Roggenkamp 1989). Fractions of about 2 ml were collected from the bottom of the tube and numbered consecutively. For SDS-polyacrylamide gel electrophoresis (SDS/PAGE) 10 μ l of each fraction was applied.

Enzyme assays. Catalase activity was measured spectrophotometrically at 240 nm by H_2O_2 degradation (Roggenkamp et al. 1974), *MOX* activity by oxidation of ABTS with H_2O_2 and peroxidase at 420 nm (Eggeling and Sahm 1978), and malate dehydrogenase by oxidation of NADH with oxaloacetate as a substrate (Bergmeyer and Bernt 1983). Measurement of β -lactamase activity was performed by monitoring degradation of the chromogenic cephalosporin Nitrocefin at 395 nm (Roggenkamp et al. 1985) and cytochrome *c* oxidase by oxidation of reduced cytochrome *c* monitored at 550 nm (Tolbert 1974). Dihydrofolate reductase activity was determined spectrophotometrically by the reduction of dihydrofolate coupled to NADPH oxidation (Schallhorn and Wilmans 1983).

Electron microscopy. To study overall cell morphology, intact cells grown in YNB plus 3% glycerol and 1% methanol were fixed in 1.5% KMnO_4 for 20 min at room temperature, poststained in 1% uranyl acetate (4–8 h), dehydrated in a graded ethanol series and embedded in Epon 812. For immunocytochemistry intact cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for 30–60 min at 0° C, dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Immunolabelling was performed on ultrathin sections, using specific antibodies against DAS, MOX or β -lactamase, by the protein A/gold method described by Slot and Geuze (1984).

Miscellaneous methods. Crude extracts of yeast cells were obtained by disruption with glass beads (0.45–0.5 mm in diameter) in a Braun homogeniser (Braun, Melsungen, FRG). Protein was quantified by the procedure of Lowry et al. (1951). SDS/PAGE (8% gels) was performed essentially as described by Laemmli (1970). For immunoblots on nitrocellulose paper the procedure described by Towbin et al. (1979) was followed. Antisera were diluted 1:500 for use. The immunoblots were finally stained with protein A-conjugated peroxidase or alkaline phosphatase conjugated with F(ab')₂ fragments of anti-rabbit IgG. Generation of rabbit antisera against purified MOX and DHFR (Roggenkamp et al. 1984) and β -lactamase (Roggenkamp et al. 1985) was done using Freund's adjuvants (Difco) as described. To obtain antisera against mouse DHFR the enzyme was overproduced under the control of the *MOX* promoter (plasmid pMED) in *H. polymorpha*. Transformed yeast cells contained about 15% DHFR relative to total cellular protein. The DHFR was purified from crude extracts by ion exchange chromatography on DEAE-Sephacel (Pharmacia) followed by gel filtration through an HPLC Ultropac Column TSK G4000SW (LKB). After this step the enzyme showed no impurities after SDS/PAGE of 1- μ g of protein and staining with AgNO_3 . Generation of antisera against DHFR in rabbits was essentially as described earlier (Roggenkamp et al. 1984).

Results

Short deletions at either end of the DAS gene abolish translocation into peroxisomes

In a first attempt to identify a putative peroxisomal targeting signal, short deletions were introduced at either end of a vector-encoded *DAS* gene. Cells of *H. polymorpha* strain LR9 were transformed and grown in the presence of 3% glycerol and 1% methanol for induction of peroxisomal proteins. Under these conditions, intact peroxisomes (harbouring enzymes for the metabolism of methanol) are not essential for viability of cells (Dion and Roggenkamp 1990).

The N-terminal deletion lacked 42 amino acids (d115) and the C-terminal deletion 45 amino acids (d82) of the *DAS* gene (Fig. 2). Expression of the truncated polypep-

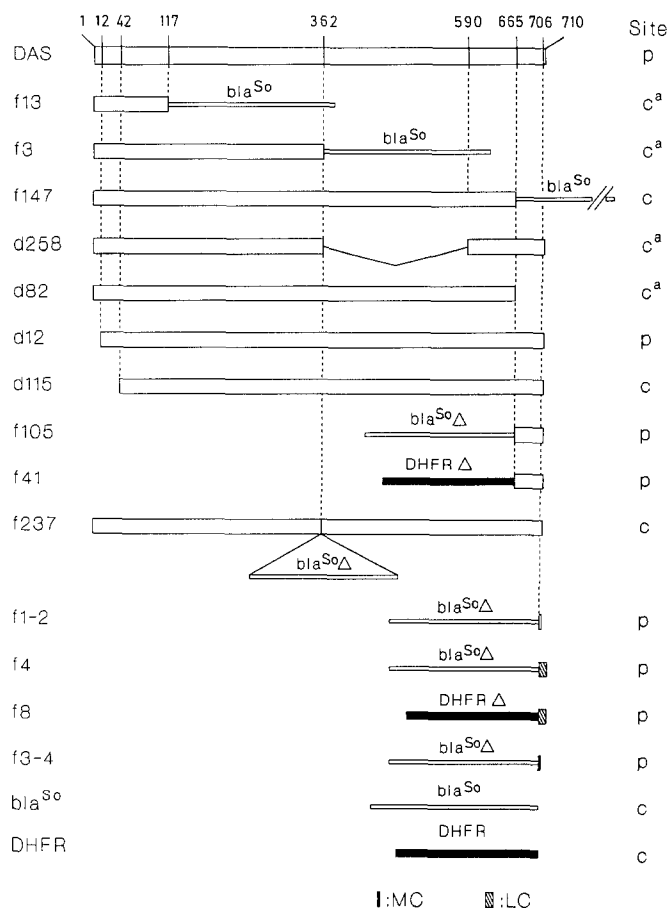


Fig. 2. Alignment of fusion proteins and truncated polypeptides. Numbers denote amino acids of dihydroxyacetone synthase (DAS), which has a total molecular mass of approximately 78 kDa. The truncated polypeptide *bla*^{So} lacks its own signal sequence of 23 amino acids and *bla*^{So} Δ lacks in addition 39 amino acids at the C-terminus. DHFR Δ lacks 5 amino acids at the C-terminus of dihydrofolate reductase. MC, peptide comprising the 4 C-terminal amino acids of methanol oxidase (MOX); LC, peptide comprising the 12 C-terminal amino acids of firefly luciferase. Intracellular site: c, cytosolic; c^a, cytosolic aggregates; p, peroxisomal. Expression of fusions and truncated polypeptides f3, f147, d258, d82 and f237 was conferred by the *DAS* gene promoter, all others by the *MOX* gene promoter. For further details see Materials and methods.

tides was verified by fractionation of crude extracts by SDS/PAGE and subsequent immunoblot analysis with a DAS-specific antiserum (not shown). Intracellular localisation of the proteins was analysed by differential centrifugation of lysed protoplasts. In contrast to the authentic DAS, the N-terminal deleted protein was not sedimentable at 15,000 g and thus had lost the ability to be imported into peroxisomes (not shown).

The C-terminal deletion sedimented into the organelle pellet. However, sucrose gradient centrifugation of this pellet showed no cosedimentation of the polypeptide with the peroxisomal fractions as revealed by comparison with the band of authentic DAS and MOX activity. The truncated DAS sedimented to a lower position, most probably as a result of aggregation of this protein in the cytosol (not shown).

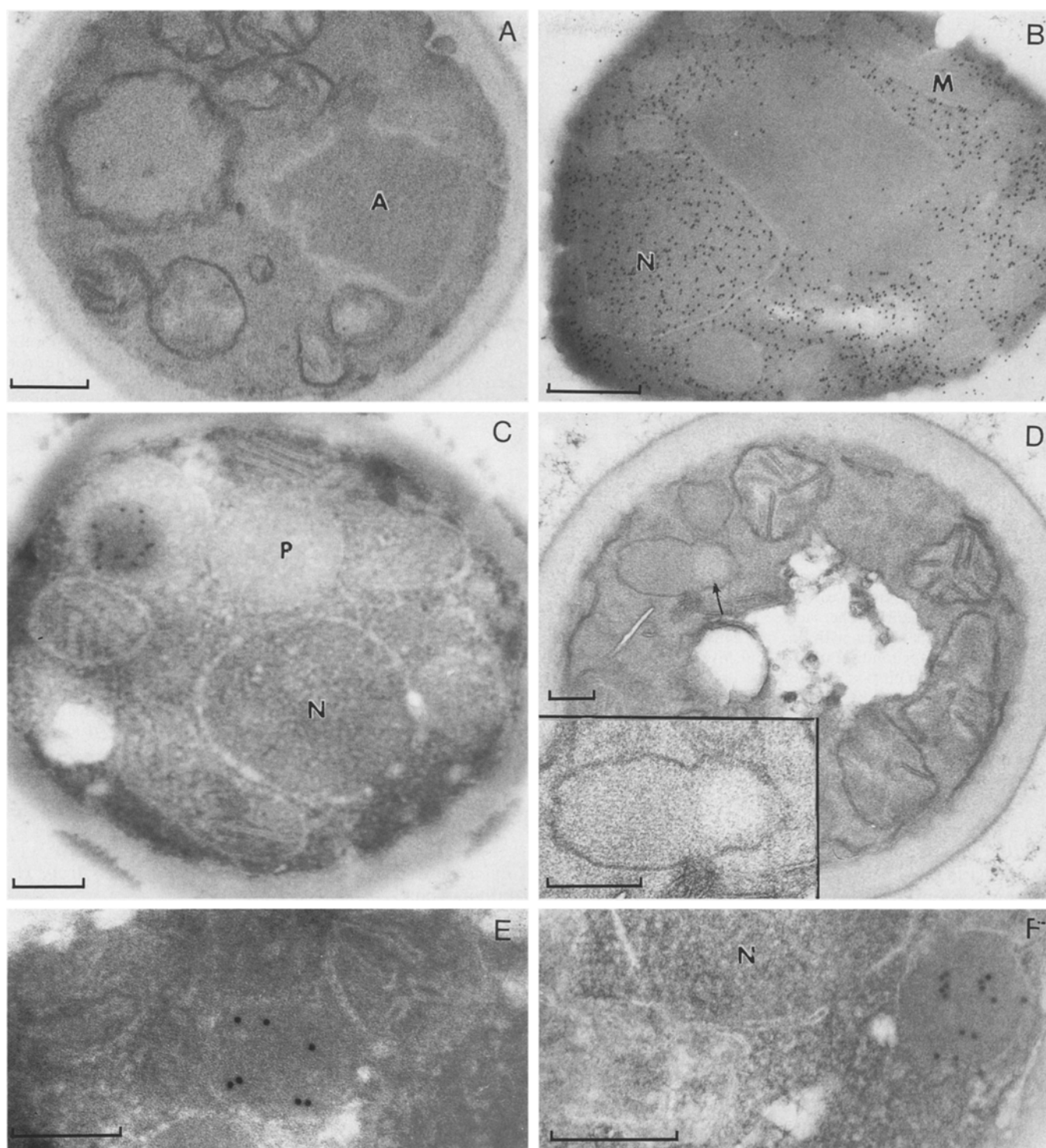


Fig. 3A–F. Thin sections of *Hansenula polymorpha* cells transformed with fusion f13 (A), bla^{So} (B), fusion f3–4 (C, D), fusion f1–2 (E) and fusion f4 (F). Yeast cells were grown as described in the legend of Fig. 4 and either fixed with KMnO₄ (A, D) or immunolabelled with a β -lactamase-specific antiserum and pro-

tein A/gold (B, C, E, F). D Arrow and inset, area of different electron density in peroxisomal matrix. A, aggregate; N, nucleus; M, mitochondrion; P, peroxisome. Bars represent 0.2 μ m. See text for further details

The N-terminus of DAS cannot mediate protein translocation into peroxisomes

Based on the assumption that the import deficiency caused by the deletions at either end of DAS reflected

the absence of putative signal sequences, we tried to achieve import of a cytosolic protein into peroxisomes by creating fusions with the terminal parts of DAS. The N-terminal part of DAS was initially tested in a gene fusion that encoded 117 N-terminal amino acids of DAS

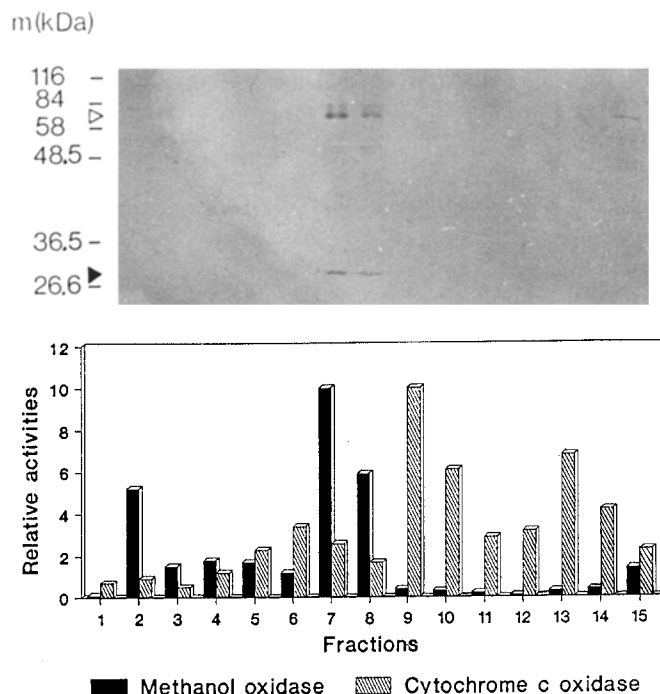


Fig. 4. Intracellular distribution of the fusion protein f105. Transformed cells were grown on Yeast Nitrogen Base (YNB) with 3% glycerol and 1% methanol as carbon sources for 48 h and subjected to cellular fractionation. The sucrose gradient for separation of organelle pellets is shown with enzyme activities of cytochrome c oxidase as a mitochondrial marker and MOX as a peroxisomal marker. For immunological analysis the proteins of the gradient fractions were separated by SDS-polyacrylamide gel electrophoresis (SDS/PAGE) (8% gels) and labelled with a DAS-specific antibody showing DAS as a peroxisomal marker (*open triangle*) and the fusion protein f105 labelled with a β -lactamase-specific antibody (*closed triangle*). See Materials and methods for experimental procedures

and the bacterial β -lactamase (lacking its signal sequence) (Fig. 2, f13). Cell fractionation studies and electron microscopy revealed large aggregates of this fusion protein (Fig. 3A) that could be labelled immunocytochemically with an antiserum against β -lactamase and protein A/gold (not shown). Labelling was evenly distributed over the cytosol including the nucleus in control cells expressing the protein bla^{So} (Fig. 3B). Aggregates were also formed in the case of the f3 protein and the construct f147 revealed a cytosolic protein (data not shown). These results indicated that N-terminal regions of DAS were not functional with respect to mediation of peroxisomal protein import.

The C-terminus of DAS (44 amino acids) enables proteins to be translocated into peroxisomes

To examine the role of the C-terminus of DAS, its last 44 amino acids were fused in-frame to the C-terminal end of β -lactamase (Fig. 2, f105). Separation of organelle pellets from transformed cells in sucrose gradients revealed that the fusion protein cosedimented with MOX, indicating association with peroxisomes (Fig. 4). Import was further demonstrated immunocytochemically after

```

2933 ACTTTGGATA CAACCCGGCA ACGATTGCCA
      F G Y N P A T I A

2963 AGAAGGTCGA AGCGTACGTC CGGGCGTGCC
      K K V E A Y V R A C

2993 AAAGAGACCC TTTGCTGCTC CACGACTTCC
      Q R D P L L L H D F

3023 TGGACCTGAA GGAAAAGCCT AACCCAGATA
      L D L K E K P N H D

3053 AAGTAAATAA GCTCTGATTA AGTAAGATGA
      K V N K L *

3083 ATAAGTTCTT TGTCTGTGAA TGC

```

Fig. 5. Revised DNA sequence of the 3' end of the *DAS* gene and deduced amino acid sequence. The amino acid sequence is altered after the histidine at position 3014–3016 compared with the formerly published sequence (Janowicz et al. 1985). The former and revised sequence are included in the EMBL Data Library under the accession number X02424. See text for further details

incubation of thin sections of transformed yeast cells with anti- β -lactamase antibodies and gold particles. Labelling was found on peroxisomal profiles (not shown) in contrast to cells expressing bla^{So}Δ, which showed the same cytosolic distribution of immunolabelling as bla^{So} (Fig. 3B).

A similar construct was created by fusing the cytosolic mouse DHFR as another passenger protein to the last 45 amino acids of DAS (Fig. 2, f41). Peroxisomal localisation of this fusion protein in transformed cells was also apparent after sucrose gradient centrifugation of organelle pellets (not shown). We concluded that the C-terminal part of DAS (44 amino acids) apparently harbours a sequence that is sufficient to direct cytosolic proteins into the peroxisomes of *H. polymorpha*.

Evidence that the extreme C-terminus of DAS from H. polymorpha represents a PTS

The results so far suggested that the C-terminal targeting signal of DAS might possibly represent a variant of the widely distributed S-K-L motif. However, no tripeptide of similar sequence was found in that region as deduced from the nucleotide sequence of the cloned gene (Janowicz et al. 1985). Upon resequencing the 3' end of the gene, a CC sequence in the reading frame had to be replaced by a single C (by a single G upon sequencing the other strand) at position 3016 (Fig. 5). The frameshift resulted in an altered polypeptide that was 8 amino acids longer than originally reported (Janowicz et al. 1985). Thus, the amino acid sequence N-K-L-COOH was deduced at the C-terminus of DAS (Fig. 5). The new sequence data start from the *ScaI* restriction site at position 2933 (Fig. 5), which was also used for the in-frame fusions f147, f105 and f41 (Fig. 2) exhibiting β -lactamase activity (f147) or DAS antigenicity (f105 and f41) (data not shown). Thus, the reading frame is correct up to the *ScaI* site and together with the sequence shown in Fig. 5 the N-K-L-COOH tripeptide can be deduced from the correct reading frame of the *DAS* gene.

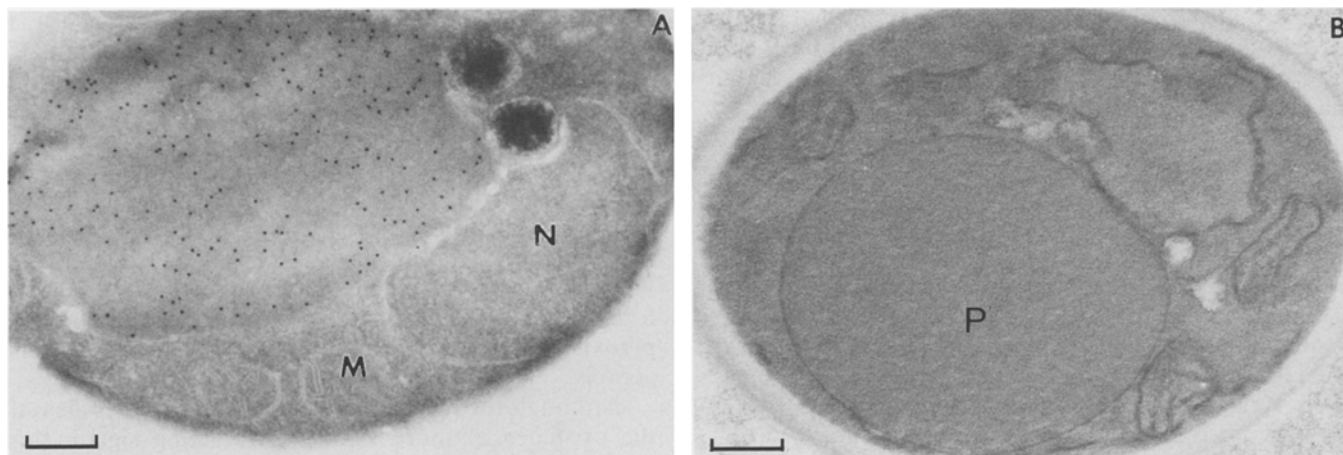


Fig. 6A and B. Enlarged peroxisomes in transformants expressing DAS with the N-terminal deletion of 12 amino acids (d12). Cells were grown as described in the legend of Fig. 4 and either fixed

in KMnO_4 (B) or labelled with a specific antiserum against DAS and protein A/gold (A). P, peroxisome; N, nucleus; M, mitochondrion. Bars represent $0.2 \mu\text{m}$

In order to demonstrate a targeting function for the tripeptide, an oligonucleotide encoding the amino acids V-N-K-L-COOH was fused to β -lactamase (Fig. 2, f1–2). Immunocytochemistry of ultrathin sections revealed a peroxisomal localization of β -lactamase (Fig. 3E). We concluded that the extreme C-terminus of DAS contains sufficient information to direct a cytosolic protein into peroxisomes.

Localization of other fusion proteins and truncated polypeptides of DAS

The results presented so far are in line with the notion that the extreme C-terminus of DAS represents a signal for peroxisomal targeting. However, structural features of this protein also seem to be critical for import. This is indicated by the finding that an in-frame insertion of the β -lactamase gene in the middle of the *DAS* gene resulted in the expression of a soluble cytoplasmic form of the fusion protein f237 (data not shown). Since this protein contained all sequence information for DAS the lack of import most probably results from changes in the tertiary structure of f237. Based on the same argument, we reasoned that large internal deletions and insertions in DAS apparently rendered these polypeptides incompetent for import. For instance, after deletion of amino acids 362–590, cytosolic aggregates of the truncated polypeptide d258 (Fig. 2) were observed (data not shown). Similarly, we reasoned that the N-terminal deletion d115 (Fig. 2) had lost its import competence. This view is also supported by the fact that a shorter deletion of 12 amino acids (d12) at the N-terminus of DAS (Fig. 2) was located in the peroxisomal matrix as shown by the generation of giant peroxisomes when it was over-expressed (Fig. 6A and B). This fact also confirms the proposal that the (extreme) N-terminus of DAS lacks a targeting function.

Peroxisomal import of β -lactamase fused to the extreme C-terminus of MOX

A deletion of 30 amino acids at the C-terminus of MOX causes cytosolic localisation of the protein in *S. cerevisiae* (B. Distel, Dissertation, University of Amsterdam 1990), which could possibly indicate a target function at this position. Although the extreme C-terminus of MOX, A-R-F-COOH, shows no homology to the N-K-L-COOH motif, we tested it for a possible target function. A synthetic oligonucleotide coding for L-A-R-F-COOH was fused to the truncated β -lactamase gene, resulting in the hybrid protein f3–4 (Fig. 2). Immunocytochemical experiments revealed a peroxisomal localization; gold particles were exclusively found in these organelles (Fig. 3C). Since the labelling was not evenly distributed over the peroxisomal matrix, the protein f3–4 obviously formed aggregates inside the organelles. In fact, a matrix area of different electron density could be observed both in KMnO_4 -fixed cells (Fig. 3D, arrow and inset) and by immunocytochemistry (Fig. 3C). The data lead to the conclusion that the extreme C-terminus of MOX comprises the peroxisomal target function.

*Function of the luciferase PTS in *H. polymorpha**

In view of the presence of an evolutionarily conserved PTS at the C-terminus of the firefly luciferase (Gould et al. 1989) and of the fact that the targeting signals of DAS and MOX are also C-terminal peptides, the PTS was tested for its function in *H. polymorpha*. A synthetic double-stranded oligonucleotide coding for the 12 C-terminal amino acids (LIKAKKGKSKL-COOH) of the firefly luciferase was fused to β -lactamase and mouse DHFR, respectively (Fig. 2, f4 and f8). Import of both fusion proteins was indicated by isolation of peroxisomes on sucrose gradients (not shown). Peroxisomal localization of the β -lactamase fusion was also demonstrated by immunocytochemistry with antibodies

against β -lactamase; gold particles were present on the peroxisomal matrix (Fig. 3F). The function of the S-K-L tripeptide in *H. polymorpha* supports the idea that it is a conserved targeting signal (Gould et al. 1990).

Discussion

Here we have described the identification of targeting signals on the two major peroxisomal matrix proteins DAS and MOX, the key enzymes of methanol metabolism in the yeast *H. polymorpha*. The signals were shown to be located at the extreme C-terminus in each protein. Their function was most clearly demonstrated by the finding that they provide sufficient information for import of cytosolic proteins into the peroxisomes. The results presented on PTSs in *H. polymorpha* are different from those obtained in studies on peroxisomal targeting in other yeast species. For the peroxisomal enzyme acyl-CoA oxidase of *Candida tropicalis* two internal fragments were shown to act independently in the import process (Kamiryo et al. 1989; Small et al. 1988). In *S. cerevisiae*, fusions of DHFR to the peroxisomal catalase A revealed that the C-terminus (and the N-terminus) of this protein is apparently devoid of a target function (Hartig et al. 1990). These data suggest that another mode of peroxisomal targeting must be considered in these yeasts. However, at least in the case of *S. cerevisiae*, this must be an additional mechanism, since both MOX and DAS were shown to undergo heterologous import into the peroxisomes of *S. cerevisiae* (Distel et al. 1987; Gödecke et al. 1989). Very recent studies on the trifunctional enzyme of *C. tropicalis* have provided significant evidence that the C-terminal tripeptide A-K-I is essential for targeting the protein to peroxisomes (Aitchison et al. 1991). In accord with our results, this signal obviously represents another PTS sequence variant.

Our experimental approach of using truncated polypeptides and fusions to cytosolic marker proteins was complicated by the fact that, in several cases, expression in transformed yeast cells resulted in an accumulation of these proteins as cytosolic aggregates. Therefore, failure of import could be explained by immediate aggregation of the translation products and by the absence of a targeting sequence. A tendency to aggregate seems to be quite common among peroxisomal protein domains. It also occurred with truncated MOX polypeptides (our unpublished results) and there have been other reports on this phenomenon. Expression of MOX in *S. cerevisiae* showed that a minor portion was imported into the peroxisomes, whereas the predominant part formed large aggregates (Distel et al. 1987). Similar observations were made with fusion proteins of DHFR with both N-terminal and C-terminal portions of the peroxisomal catalase A in *S. cerevisiae*; only cytosolic aggregates were detected (Hartwig et al. 1990). The reasons for the frequent occurrence of these aggregates are still unknown.

Apart from protein aggregation, import competence is apparently related to the three-dimensional structure of polypeptides, as has clearly been shown for proteins

destined for the mitochondria (Chen and Douglas 1987; Eilers et al. 1988). In line with this view, the fusion f237 probably failed to be imported, although it contains all primary DAS sequence information.

Studies on different protein transport systems strongly suggest the participation of specific proteins in the folding process to ensure a loose conformation facilitating membrane translocation (Chirico et al. 1988; Crooke and Wickner 1987; Deshaies et al. 1988; Pelham 1989; Rothman and Kornberg 1986; Wickner 1989). Therefore, tertiary structures and certain protein domains on peroxisomal proteins may be important for these interactions.

Although the results discussed above cannot rule out the existence of more than one targeting signal, this seems very unlikely in view of the fact that the identified peptide stretches of MOX and DAS are sufficient to direct cytosolic proteins to peroxisomes followed by translocation into the matrix. These findings are in full agreement with the properties of the S-K-L-COOH motif of the firefly luciferase (Gould et al. 1989). It should be noted, however, that we used the four C-terminal amino acids of DAS and MOX in fusions f1–2 and f3–4, respectively. The rationale behind this was to maintain at least one amino acid in the original context, though there is no evidence at present as to whether this is important for the target function or not. The use of passenger proteins generally encounters the problem that the context amino acids are arbitrary and that the efficiency of import can scarcely be estimated. The elucidation of this point has to await studies with an authentic peroxisomal protein in which amino acids close to the signal are altered and import efficiency is subsequently estimated. This could be done with MOX for example, by monitoring growth on methanol, which depends on import of the protein, in a MOX-negative mutant (Roggenkamp et al. 1989).

Several lines of (in)direct evidence are in accord with the notion that the targeting signals in *H. polymorpha* are indeed tripeptides. Firstly, the well studied PTS S-K-L-COOH of firefly luciferase (Gould et al. 1990) is functional in this yeast. Secondly, the N-K-L-COOH motif of DAS can be derived from the PTS by a single amino acid exchange. Thirdly, the A-R-F-COOH motif of MOX can similarly be derived from the PTS by a single amino acid exchange, given the fact that the PTS, based on tests of its function in mammalian cells, was shown to be degenerate to a certain extent, as indicated by the consensus sequence of amino acids S-,A-,C-/K-,H-,R-/L-COOH (Gould et al. 1989). A common feature of all tripeptides described so far is also the presence of a hydrophobic residue at the third position and a basic amino acid at the second position (Roggenkamp 1992). In summary, the described targeting signals of *H. polymorpha* are likely to be novel variants of a C-terminal tripeptide.

Strong variation in the primary sequence is common for signal sequences that sort proteins to cell organelles other than peroxisomes. In general, they comprise fairly large amino acid stretches allowing the formation of secondary structures (von Heijne 1985, 1986; von Heijne

et al. 1989) that most probably contain the targeting information. In contrast, short amino stretches, such as for example the recently identified ER retention signals for luminal ER proteins comprising four C-terminal amino acids, show a marked sequence homology (Pelham 1986). Comparative studies on such signals in the yeasts *S. cerevisiae* and *Kluyveromyces lactis* revealed that a single amino acid exchange also determines the specificity of the retention (Lewis et al. 1990). Therefore, the pronounced variation in the three peroxisomal targeting signals in *H. polymorpha* may suggest the existence of more than one putative receptor. Final clarification of this point has to await the identification and characterisation of such components for peroxisomal protein sorting. These investigations should now be feasible based on the described properties of the peroxisomal targeting signals.

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